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EXTRACTION AND CHARACTERIZATION OF EXTRACELLULAR POLYSACCHARIDE FROM RALSTONIA SOLANACEARUM CAUSING WILT OF TOMATO

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ABSTRACT

Extracellular polysaccharide (EPS) is a one of the most important virulence factors involved in wilting of plants by Ralstonia solanacearum Field survey was undertaken in major tomato growing districts of the Karnataka to isolate and identified the *R. solanacearum* from wilted tomato plants and soil samples. A total of 100 isolates were isolated, ten highly virulent isolates were selected based on morphological, biochemical characteristics and pathogenicity studies, as well as 16S rRNA gene sequencing. The wilt influencing factor of EPS produced from isolates of *R.* solanacearum was studied by using the phenol-H_2SO_4 method. Among ten isolates, isolate number-5 (RS5) was found to be potential isolate for EPS production (840mg/l). EPS production of RS5 was optimized in Casamino acid Peptone Glucose (CPG) broth for different environmental and nutritional conditions. The optimum temperature, pH, and NaCl concentrations for maximum EPS production (840, 820 and 789 mg/l) was found to be 7.0, 30 °C and 80 mM respectively. Sucrose was found to be the suitable carbon source to produce maximum yield of EPS (800 mg/l) and 3 days of incubation period was found to be production of maximum yield of EPS (820 mg/l). The Fourier Transform Infrared Spectrometer (FT-IR) revealed typical characteristics of EPS. The FTIR spectrum of EPS showed a broad intense band at 4000 cm^{-1} and found stretching at 2933.53 cm^{-1} (-OH) 1662.52 cm^{-1} ($-CH_3$), and 1080.78 cm^{-1} (-C=O) corresponds to suggests the presence of broad stretching of functional groups of EPS. The strongest absorption bands appear at about 1080cm^{-1}, which was described as C-O stretching in carbohydrate. The present work demonstrates for the production and partial characterization of extracellular polysaccharide from R. solanacearum.

KEYWORDS: Ralstonia, Solanacearum, Bacterial, Wilt, Extracellular, Polysaccharides, TLC, FTIR, Tomato

INTRODUCTION

Ralstonia solanacearum is a non sporulating gram-negative soil-borne bacterium that causes bacterial wilt disease in diverse and important food crops, such as tomato, potato, banana and ginger (Hayward, 1991). The *R.* solanacearum can survive for long periods of time in a nutrient-depleted environment (Grey *et al.*, 2001; Shanmugam *et al.*, 2004). *R.* solanacearum generally invades its host through wounds in the roots, colonizes the xylem vessels, and spreads rapidly up the stem and through the plant. The characteristic wilting symptoms result from an excessive production of extracellular polysaccharides (EPS) within the vascular system, thus altering water fluxes in the plant (Genin *et al.*, 2005). The pathogenesis of R. solanacearum is mainly due to the gradual mechanical blocking of plant vessels by the bacterium itself and its surrounding slime of EPS (Buddenhagen and Kelman, 1964). Avirulent *R.* solanacearum strains lack of EPS.

Recent studies confirmed the role of EPS as the primary cause of pathogenicity (Denny and Baek, 1991; Kao *et al.,* 1992).

In nature, biopolymers often play important roles in maintaining cell feasibility by conserving genetic information, by storing carbon-based macromolecules, by producing energy or reducing power and by defensive an organism against attack from dangerous environmental factors (Kazak *et al.,* 2010). Extracellular polysaccharides (EPS) may be connected to the bacterial cell as a capsule, be produced as fluidal slime, or be present in both forms (Denny, 1999). EPS play a significant role in pathogenesis of many bacteria by both direct interference with host cells and by providing resistance to oxidative stress. EPS1 is the chief virulence factor of the bacterial wilt disease caused by *R.* solanacearum in solanaceous crops, since *eps* mutants were severely reduced in systemic colonization of tomato plants when introduced through unwounded roots and did not produce typical wilt symptoms even when directly inoculated into stem wounds (Milling *et al.,* 2011).

Microbial polysaccharides are high molecular weight carbohydrate polymers present either at the external membrane as lipopolysaccharides (LPS) that mainly determine the immunogenic property or secreted as capsular polysaccharides (CPS) forming a discrete surface layer (capsule) associated with the cell surface or excreted as EPS that are only loosely connected with the cell surface (Cuthbertson *et al.,* 2009). Virulent strains of R. *solanacearum* possesses varied genes involved in colonization and wilting of host plants, such as those coding for lytic enzymes, EPS, hypersensitive response and pathogenicity (*hrp*) genes, encoding structural protein genes injected by a type III secretion system (T3SS) from the pathogen into the plant cell, genes coding for factors implicated in cell adherence and others (Denny et al., 1990). R. solanacearum produces a variety of extracellular products that contribute to its ability to colonize host plants and cause disease symptoms. One of the most important of these is, high molecular mass extracellular polysaccharide EPS. In planta studies of EPS deficient mutants, (Kao *et al.,* 1992) suggest that EPS is the cause of wilting in infected plants, as it blocks the vascular system, thereby alters water movement and producing wilt symptoms (Denny, 1995). EPS production, particularly its role in pathogenesis as determined through transposon mutagenesis, has been explored most extensively in R. solanacearum (Denny and Baek, 1991). Several phytopathogenic bacterial species produce high amounts of EPSs either in pure culture or during *in* planta multiplication. Even though usually related to pathogenicity, it is often difficult to know if the EPSs take active part in symptom production or if they indirectly favour infection (Boucher *et al.,* 2001). In *R.* solanacearum, it has been reported that all virulent wild-type strains (mucoid colonies) produce EPS (Buddenhagen and Kelman, 1964; Poussier *et al.,* 2003), while EPS-deficient mutants (non-mucoid colonies) are avirulent. R. solanacearum EPS appears to be highly heterogeneous, since it has a varying composition among strains (Drigues *et al.,* 1985).

A variety of extracellular products that contribute to its ability to colonize host plants and cause disease symptoms are produced by R. solanacearum. According to Genin and Boucher (2002), one of the most important extracellular products is an acidic, high molecular mass, extracellular polysaccharide (EPSI). However, EPSI mutants were still pathogenic, poorly colonizing the stem, insinuating that EPS might be responsible for minimizing plant recognition of the bacterial surface structures (Denny *et al.,* 1998; Schell, 2000). EPSI is more than 90% of the total R.solanacearum EPS produced, and approximately 85% appears as a released, cell-free slime, whereas 15% has a cell surface-bound capsular form. *In* planta, EPS would probably act by occluding xylem vessels, interfering directly with normal fluid movement of the plant, or by breaking the vessels due to hydrostatic overpressure. On the other hand, EPSI might also favour stem colonization by the pathogen, since EPSI-deficient mutants were shown to multiply more slowly, and colonized poorly the

stem of infected plants (Saile *et al.*, 1997). In that sense, EPSI would be contributing to minimizing or avoiding the recognition of bacterial surface structures such as pili and/or lipopolysaccharide by plant defence mechanisms. As EPS-deficient mutants can infect and multiply to some extent *in* planta without inducing wilting symptoms, EPS might take part mainly in late stages of the process, modulating disease severity rather than the infective ability of the bacterium. In *R.* solanacearum, EPS is thought to be the main factor accounting for the virulence of the pathogen (Hikichi *et al.,* 2007). The main objective of the present study was to extraction and characterization of extracellular polysaccharide from *R.* solanacearum causing wilt of tomato

MATERIALS AND METHODS

Isolation and Identification of R. Solanacearum

Field surveys were conducted in major tomato growing districts of Karnataka, India. The suspected plant material and soil samples were collected from the field survey, brought to the laboratory and isolated the target pathogen from the diseased plants. Plant materials were surface sterilization with 70% ethyl alcohol followed by three repeated washings with distilled water and blot-dried and the plant parts (0.5–1 cm) were placed on petriplates containing Kelman's TZC agar medium. Plates were incubated at 28 ± 2 °C for 24–48 h (Kelman 1954; Elphinstone *et al.* 1998). The virulent (white fluidal irregular colonies with pink centre) isolates were observed for colony characteristics, biochemical, physiological, Biovar, hypersensitive and pathogenicity tests for confirmation of the pathogen (Vanitha et al., 2009; Narasimha Murthy *et al.,* 2012). The PCR product was sequenced by Sanger di-deoxy method. Nucleotide BLAST was performed to all the ten obtained sequences in NCBI (www.ncbi.nlm.nih.gov) using blastn suite and top 5 hit sequences with more than 98% similarity to the query sequences were selected for further phylogenetic analysis. Multiple sequence alignments of all these sequences were performed by using CLUSTAL-X software version 2.1. Phylogenetic tree was constructed and the alignment data was analyzed by neighbor-joining (NJ) method. The sequences were deposited in NCBI GenBank.

Growth and Maintenance of *R.* Solanacearum Strains

Ralstonia solanacearum isolates were cultured in Casamino acid Peptone Glucose (CPG) broth (1 g of Casamino acids; 10 g of peptone; 5g of glucose in 1000 ml of distilled water) (Kleman, 1954) at 28±2 °C. Antibiotics, where used, were at the following concentrations; Kanamycin 50µg/ml; tetracycline 15µg/ml; ampicillin 100µg/ml; streptomycin 25µg/ml.

Pathogenicity Testing of Isolates

Virulence analyses of the isolates were carried out on a bacterial wilt susceptible tomato cultivar, Arka Meghali was procured from IIHR Bangalore, India. The 20 days old healthy seedlings were selected and used for pathogenicity assay (Narasimha Murthy *et al.,* 2012). Bacterial inoculum was prepared in CPG broth and pelleted by centrifuging at 12,000 rpm for 10 min, suspensions were prepared in sterile distilled water and spectrophotometrically adjusted to OD600nm=0.1 (approximately 1×10^{8} CFU/ml) (Ran *et al.,* 2005). The root system of each plant was wounded with a sterile scalpel and 5 ml of inoculum per plant was poured on the wounded root system. Plants were placed in a greenhouse and observed daily (Williamson *et al.,* 2002). Observations were made from one week after pathogen inoculation. If the plant showed typical wilt symptoms, the interaction was considered as pathogenic. The isolates were categorized based on pathogenicity into 4 group's viz., highly pathogenic, moderately pathogenic, weakly pathogenic and non pathogenic in the tested tomato variety, whereas non inoculated tomato seedlings were served as control (Tans *et al.*, 2001).

Separation and Purification of the Extracellular Polysaccharides

The virulent isolates of *R.* solanacearum were cultured in the optimal EPS producing medium at 28 ± 2° C for 24–72 h. Production was carried out in 250 ml flasks containing 50 ml of medium. After removal of the cells by centrifugation (7000 rpm in, 10min), the EPS was precipitated over night by adding three fold volume of cold ethanol (96%) and left overnight at 4 °C Smitinont *et al.,* (1999). The cell suspension was centrifuged at 20,000 rpm in 20 min, at 4 °C. This technique was believed not to cause cell lysis (Liu and Fang, 2002). The supernatants were filtered through a 0.22 μm membrane. The filtrate was used as the EPS sample. The EPS pellets were dried at 55°C for 12 h and the dry weight of each was measured (Jeong *et al.,* 2008). Means and standard errors were calculated from three independent experiments. The protein content of EPS was determined by the Bradford method (1976) with bovine serum albumin as the standard.

Estimation of Polysaccharide by Spectrophotometry

The dissolved bacterial polysaccharide solution was used for estimation of EPS by Phenol sulphuric acid method following Dubois *et al.* 1956. This method was also applied to determine the total amount of carbohydrates in the food. It is significant to study carbohydrate content because the absorbtivity of different carbohydrates varies. The carbohydrates in the sample will react with phenol to produce a yellow-gold color, which can then be measured using a spectrophotometer. Reaction mixture in a test tube contained 1 ml of EPS solution and 1 ml of aqueous phenol and 5 ml of conc. H_2SO_4 was added to it. After vigorous shaking, the tubes were allowed to stand for 20 min. The absorbency was measured at 490 nm. EPS solution in distilled water was used as control. The amount of EPS was determined against glucose standard.

Optimization of Exopolysaccharide Production

The present work was carried out to optimize the nutritional and environmental parameters for improving EPS production by *R.* solanacearum strains. The CPG broth was prepared with 4% of each glucose, lactose, fructose, sucrose, mannitol and maltose were added to find the effect of carbon sources on the EPS production, pH range 6-8 was adjusted using 0.1 N HCL and 0.1 N NaOH were performed to find the effect of pH and different temperature at 20-40 °C and salt concentrations are 80-20 mM NaCl for the production of EPS. In incubation period the flasks were incubated at 28± 2 °C for 5 days and every 12 hours the culture supernatant was precipitated with ice cold ethanol (1:3) ratio, dried and the biomass were weighed and expressed in (mg/L) (Sunil *et al.,* 2013; Krithiga *et al.,* 2014).

Thin Layer Chromatography (TLC) Analysis of EPS

The extracted polysaccharides were hydrolysed in 2 mol HCl for 20 h at 100 °C.Samples were vacuum dried at 40 °C and resuspended in deionized water The component sugars were qualitatively identified by thin layer chromatography (TLC), using silica gel chromatography plates. According to the method provided by Wang et al (1999).The final extraction solution of the product, EPS was spotted, spread, colorized, and the purity was qualitatively analyzed.

Fourier Transformed Infrared Spectroscopy Analysis of EPS

After running TLC, samples were scrapped off from the plates, pooled and eluted with distilled water and dried to get the compounds of interest in a solid from. Fourier Transformed Infrared Spectroscopy (FTIR). The major structural groups of the purified EPS were detected using FTIR was used to determine the functional groups and the chemical bonds present in the biologically active fraction of the EPS and thus determine its chemical nature (Helm *et al.,* 1995). Pellets for infrared analysis were obtained by grinding a mixture of 2 mg of exopolysaccharide with 200 mg of dry KBr, followed by

pressing the mixture into a 16-mm diameter mold and infrared spectrum was recorded in the region of 4,000-400 cm^{-1}, using FTIR Spectroscopy (Shimadzu Corporation).

RESULTS

Isolation and Identification of the *R.* Solanacearum

After incubation, the pink centers with white fluid colonies were selected and a total of 100 strains of *R.* solanacearum were isolated, identified and stored as pure cultures on TZC slants at 4 °C for further studies. Microscopic studies revealed that isolates were Gram negative, rod shaped, non spore forming and it was confirmed by standard biochemical tests (Figure 1) (Narasimha Murthy *et al.*, 2012). The identification of the *R. solanacearum* isolates was confirmed by molecular method. The BLAST analysis of the sequences showed 98% to 99% identity to several isolates of R. solanacearum strains. Among 100 isolates, ten highly virulent strains were characterized and were identified as *R.* solanacearum, RS1, RS2, RS3, RS4, RS5 RS6, RS7, RS8, RS9 and RS10 with Gen bank Accession numbers KF924739, KF924740, KF924741, KF924742, KF924743, KF924744, KF924745, KF924746, KF924747and KF924748 respectively. Phylogenetic tree was constructed and the alignment data was analyzed by neighbor-joining (NJ) method (Figure 2).

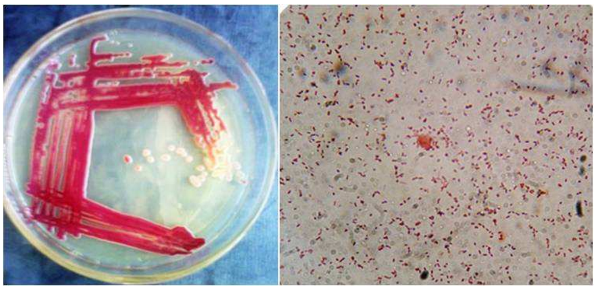

Figure 1: Colonies of Ralstonia Solanacearum from Infected Tomato Fields and Microscopic View of *R.* Solanacearum

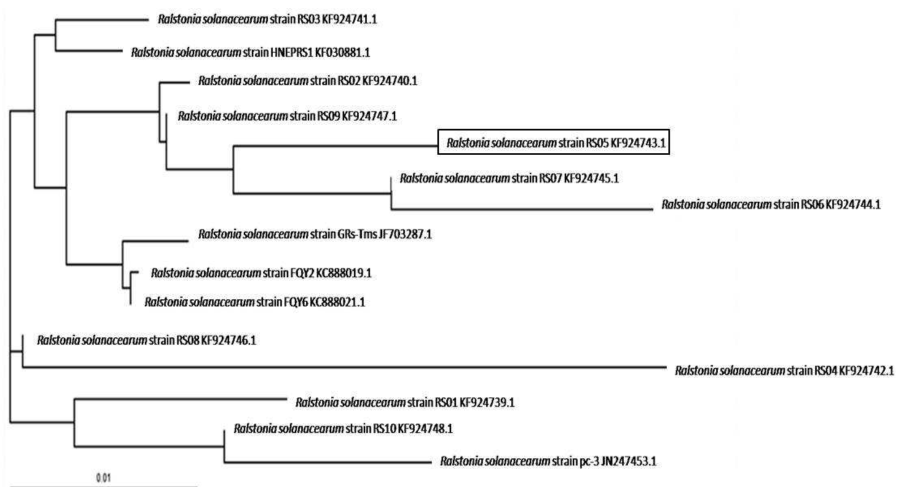

Figure 2: Phylogenetic Relationships of *R.* Solanacearum Isolates Inferred by Neighbor Joining (NJ) Bootstrap Tree Analysis of 16S rRNA Sequences

Pathogenicity Testing of R. Solanacearum

Pathogenicity was confirmed by the development of wilt symptoms on tested tomato plants after 7 days of pathogen inoculation followed by reisolation and identification of the causal organism from diseased plants. Based on the development of wilt symptoms, R. solanacearum isolates were grouped into highly pathogenic, moderately pathogenic,

weakly pathogenic and avirulent (Figure 3).

Figure 3: The Symptomatical Variation on Tomato Plants of 1-5 Scale Studied Under Green House Conditions 1: No Symptoms, 2: Slight Chlorosis, 3: Moderate Chlorosis, 4: Severe Chlorosis, 5: Plant Death

Separation and Purification of the Extracellular Polysaccharides

The purpose of this testing was to obtain the efficient *R.* solanacearum isolated from wilted soil and plant samples which produce high amount of EPS. Among 100 isolates only ten isolates were screened for EPS producing activity based on the pathogenicity assay. Out of ten isolates, isolate number-5 (RS5) (Accession number- KF924743) produced higher 840mg/L of exopolysaccharide as compared to other pathogens (Figure 4). A chemical investigation of the polymer reveals the presence of proteins and carbohydrates. The protein content in EPS was in the range of 9.5%. Whereas, carbohydrates accounted for more than 70%

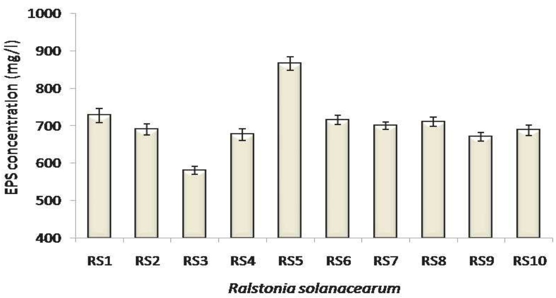

Figure 4: Screening of *R.* Solanacearum Isolates for EPS Production

Optimization of Exopolysaccharide Production

Optimized parameters for efficient EPS production are; Incubation period (in days) 3; Temperature 30 °C; Carbon source sucrose; Sucrose concentration 4%; pH 7.0 and Salt (NaCl) concentration in mM 80mM. The production of EPS by *R.* solanacearum (RS5) at different temperatures during the experiment, the maximum EPS (840mg/l) produced at 30 °C after incubation. The different pH during the experiment, the maximum EPS (820 mg/l) produced at pH7. Extracellular polysaccharide production was influenced by different sugars and the maximum EPS production (800 mg/l) was obtained while sucrose as a carbon source and the next were glucose, fructose, lactose, maltose and mannitol. The production of EPS from different days of incubation, the maximum (820 mg/l) produced at third day, compared to other days of incubation period (Figure 5). The EPS production was found from different NaCl concentrations, maximum EPS (789 mg/l) produced at 80mM salt concentration while decreased the EPS production when increased the salt concentration up to 200 mM (Figure 6).

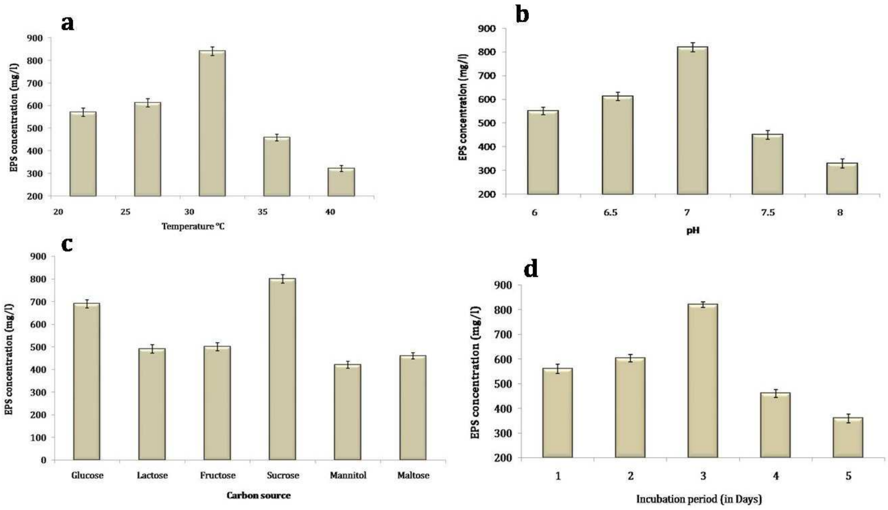

Figure 5: Optimization of Exopolysaccharide Production at Different Environmental Conditions. A: Production of EPS from Incubation Temperature; B: Production of EPS From Different Ph; C: the Influences of Different Sugars on the Production of EPS from *R.* Solanacearum (RS5) Isolate; D: Production of EPS from Incubation Period in Days

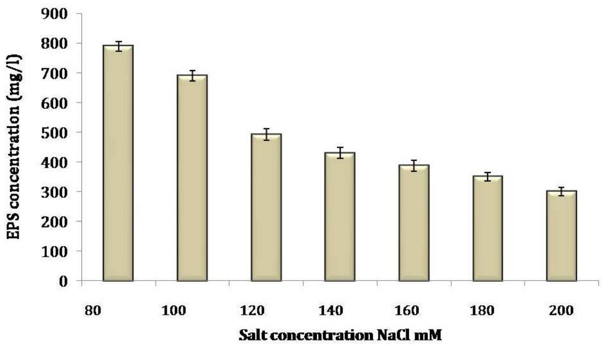

Figure 6: The Influences of Different NaCl Concentrations on the Production of EPS from R. Solanacearum (RS5)

Thin Layer Chromatography (TLC) of Extracellular Polysaccharide

The EPS produced by *R.* solanacearum was evaluated by TLC analysis. A dark oval-shaped glucose (standard) spot was seen in the lane 1 of the plate and remaining lanes were identical extracted EPS (Figure. 7). The result of TLC was shown that the purified EPS in the experiment achieved the preferred purity and was appropriate to make further analyses on its chemical property and structure.

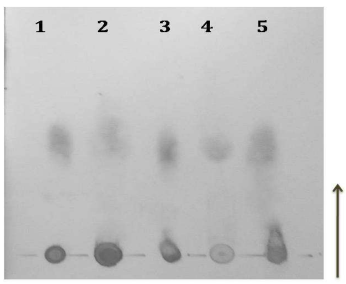

Figure 7: The Thin Layer Chromatography for Extracellular Polysaccharide. 1: Standard (Glucose); 2–4: All Identical Extracted EPS Samples

Fourier Transformed Infrared Spectroscopy Analysis of EPS

Functional group was detected by using FTIR spectrum from crude EPS. Each peak represents a specific molecular movement and includes the parts of functional groups from which they are derived. The data indicates the presence of functional groups similar to those present in EPS. FT-IR spectrum of the purified extracellular polysaccharide found stretching at, 2933.53 cm^{-1} (-OH) 1662.52 cm^{-1} ($-CH_3$), and 1080.78 cm^{-1} (-C=O) (Figure 8). The IR spectrum of polymer proved the presence of carboxyl group which may serve as a binding site for divalent cations. This carboxyl group may also work as a functional moiety to generate an inventive new or modified polymer, by using different new approaches like synthetic polymers.

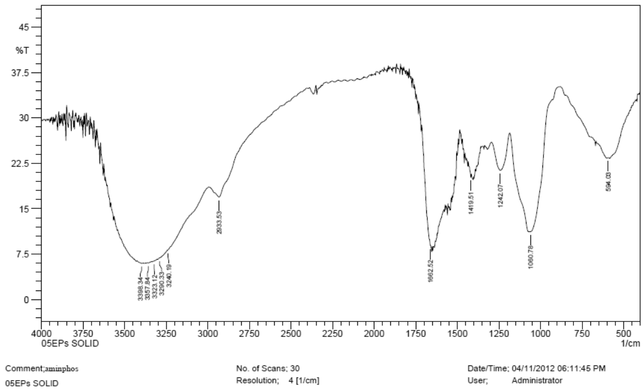

Figure 8: FTIR Spectroscopy Analysis of EPS. Important Peaks Observed; Absorbance Peaks Used for Spectral Analysis

DISCUSSIONS

Carbohydrates are the major component of the cytoskeleton and an important nutritional requirement for the growth and cell development (Richert *et al.,* 2005). The purpose of this experiment was to obtain the efficient *R.* solanacearum strains isolated from wilted soil and tomato plant samples which produce high amount of EPS. Ten isolates were screened for EPS producing activity, out of ten isolates, isolate RS5 produced higher amount of exopolysaccharide as compared to other isolates. Identification of isolate RS5 was confirmed by their molecular characterization. Therefore isolate RS5 was used for the further work. The effect of different carbon sources on the production of EPS was studied. In this study, the sucrose was the suitable carbon source for the EPS production. Sucrose at 4% concentration in medium showed higher EPS production. The possible reason is that EPS could have been gradually decomposed into monosaccharides and compensated as carbon source for continuous growth in *R.* solanacearum culture. The influence of the carbon source on the production and composition of the EPS has been reported by other authors as well (Kanmani *et al.,* 2011; Chang *et al.,* 2011, Jin *et al.,* 2003). The EPS production in *R.* solanacearum growth is influenced by many factors. It was proven in the experiment that the EPS production is higher when Sucrose is used as a carbon source than any other sugar that was used, which is in accordance with similar domestic and foreign reports.

The bacterial biomass was the highest when the maximum EPS production was achieved. The EPS production decreases quickly with the prolonging of time and the declining of pH, especially under 30°C. The bacteria cultured under a lower temperature have a prolonged logarithm and stable period (Zisu and Shah, 2003). The highest production at 30°C, the reasons for earlier production peak and quicker production decline under 40°C may be that it is more suitable for the enzyme activity under 30 °C and the metabolism rate of the polysaccharide is quick. Therefore, it is better for the EPS

collection to be under 30°C from the point of polysaccharide accumulation. Then, we can conclude that the best condition for *R.* solanacearum to produce polysaccharide is by incubation at 30°C for 24–72 h. The EPS production was found to higher at 80mM salt concentration. Finally, the effective method for extracting EPS was explored in this experiment. It was shown by using thin layer chromatography that our experimental method is reasonable and effective. The successful method for extracting EPS was explored in this trial. It was shown by using thin layer chromatography that our experimental method is reasonable and effective. However, in the separation and purification of EPS, protein and other charged polysaccharides can mutually combine through the complicated chemical bonds, which bring about the difficulties for separation and purification of EPS (Li and Xia, 2003).

Functional group was detected by using FTIR spectrum from crude EPS. The IR spectrum of polymer has shown a broad intense band at 4000 cm^{-1}-400 cm^{-1}. The sharp band at 2933.53 cm^{-1} strongly suggests a presence of broad stretching (O-H, carboxylic acid and H- bonded) groups. The peak at 1080.78 cm^{-1} corresponds to stretching of (C-O, alcohol, ether and phenol) groups. Polysaccharides possessing carboxyl group is reported previously (Jindal *et al.*, 2010). The IR spectrum of polymer proved the presence of carboxyl group which may serve as a binding site for divalent cations. The carboxyl groups may also work as functional moieties to generate new and/or modified polysaccharide variants using different approaches including polymer engineering or novel formulation designing by linking this polysaccharide with starch and/or other synthesized polymers (Ha *et al.*, 1991).

The EPS from *R.* solanacearum may act as a defensive coating that prevents bacteria from agglutination by divalent cations in intercellular spaces or fixation onto plant cell walls through the binding between LPS and lectins, after leaf infiltration (Sequeira and Graham, 1977). The presence of hydroxyl groups within the polysaccharide favored the possibility of hydrogen bonding with one or more water molecules. Thus, the polymer could swell and even dissolve partially or completely in water (BeMiller *et al.*, 1996). Further, the insolubility of the polysaccharide in organic solvents can be explained by the fact that the increased number of hydroxyl groups present in the polymer increases the crystalline of the polymer and these forces are difficult to break by organic solvents (James, 1986).

CONCLUSIONS

A new extracellular polysaccharide was produced from *R.* solanacearum. This study will open doors for further studies on attaining a greater production of EPS from *R.* solanacearum and also for clarifying their exact composition, structures, and biological activities in the host. The exopolysaccharide production was optimized under different nutritional and environmental conditions. EPS production was found to be maximum in CPG broth in presence of 4% sucrose as a carbon source and 80mM salt concentration, temperature at 30 °C within three days of incubation. A chemical analysis of the EPS reveals the presence of proteins and carbohydrates. EPS was characterized by partially FTIR spectrum, which shows the presence of carboxylic acid group and H- bonded group. Further studies are needed to evaluate the mechanism of virulence factor exopolysaccharides in wilted tomato plant.

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